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TO ALL WHOM IT MAY CONCERN:

Be it known that WE, GEORGES FREYSSINET, RICHARD DEROSE, and JULES HOFFMANN, citizens of France, the United States, and France, respectively, all residing in France and whose post office addresses are 21, rue de Nervieux, F-69450 Saint Cyr au Mont d'Or, France, 216, rue de Saint Cyr, F-69009 Lyon, France, and 5, rue Closener, F-67000 Strasbourg, France, respectively, have invented an improvement in

GENE CODING FOR ANDROCTONINE, VECTOR CONTAINING IT AND DISEASE-RESISTANT TRANSFORMED PLANTS OBTAINED

of which the following is a

SPECIFICATION

## CROSS REFERENCE TO RELATED APPLICATIONS

**[0001]** This application is a national stage of International Patent Application Serial Number PCT/FR98/01814, filed August 18, 1998, which claims priority from French Patent Application Serial Number FR 97/10632, filed August 20, 1997, the contents of which are incorporated herein by reference in their entireties.

INTRODUCTION

**[0002]** The present invention relates to a DNA sequence coding for androctonine, to a vector containing it for the transformation of a host organism and to the process for transforming the organism.

**[0003]** The invention relates more particularly to the transformation of plant cells and plants and to the androctonine produced by the transformed plants, giving them resistance to diseases, in particular diseases of fungal origin.

**[0004]** There is today an increasing need to make plants resistant to diseases, in particular fungal diseases, in order to reduce, or even avoid altogether, the need for treatments with antifungal protection products, in order to protect the environment. One means of increasing this disease-resistance consists in transforming the plants so that they produce substances capable of defending themselves against these diseases.

**[0005]** Various substances of natural origin are known, in particular peptides, which have bactericidal or fungicidal properties, especially against the fungi responsible for plant diseases. However, the problem consists in finding such substances which not only can be produced by transformed plants, but also can conserve their bactericidal or fungicidal properties and confer these properties to the plants. For the purposes of the present invention, the terms bactericidal and fungicidal are understood to refer both to the actual bactericidal or fungicidal properties and to the bacteriostatic or fungistatic properties.

**[0006]** Androctonines are peptides produced by scorpions, in particular from the species *Androctonus australis*. An androctonine and its preparation by chemical synthesis are described by Ehret-Sabatier *et al.*, along with its *in vitro* antifungal and antibacterial properties.

[0007] The androctonine genes have now been identified, and it has also been found that they can be inserted into a host organism, in particular a plant, in order to express an androctonine, both for the preparation and isolation of this androctonine and to give the host organism properties of resistance to fungal diseases and to diseases of bacterial origin, thereby providing a particularly advantageous solution to the problem outlined above.

## BRIEF DESCRIPTION OF THE DRAWINGS

[0008] **FIGURE 1** shows the schematic structure of plasmid pRTL-2 GUS.

[0009] **FIGURE 2** shows the schematic structure of expression cassette created through the replacement of the *NcoI/Bam*HI fragment from pRTL-2 GUS by a similar fragment encoding the PR-1a-androctonine fusion protein.

[0010] **FIGURE 3** shows the schematic structure of the multiple cloning sites contained within the *Agrobacterium tumefaciens* vector plasmid.

[0011] **FIGURE 4** shows the schematic structure of The schematic structure of pRPA-RD-174.

[0012] **FIGURE 5** shows the schematic structure of the plasmid pRPA-RD-184.

## DETAILED DESCRIPTION OF THE INVENTION

[0013] The subject of the invention is thus, firstly, a nucleic acid fragment coding for an androctonine, a chimeric gene comprising the fragment coding for an androctonine and heterologous regulation elements in positions 5' and 3' which can function in a host organism, in particular in plants, and a vector for transforming host organisms containing this chimeric gene, and the host organism transformed. The invention also relates to a transformed plant cell containing at least one nucleic acid fragment coding for an androctonine, and to a disease-

resistant plant containing the cell, in particular a plant regenerated from this cell. Lastly, the invention relates to a process for cultivating transformed plants according to the invention.

[0014] According to the invention, the term androctonine is understood to refer to any peptide which can be produced by and isolated from scorpions, in particular from the species *Androctonus australis*, these peptides comprising at least 20 amino acids, preferably at least 25, and 4 cysteine residues which form disulfide bridges between themselves. Advantageously, the androctonine essentially comprises the peptide sequence of general formula (I) below:

Xaa-Cys-Xab-Cys-Xac-Cys-Xad-Cys-Xae (SEQ ID NO.:15)

(I)

in which Xaa represents a peptide residue comprising at least 1 amino acid, Xab represents a peptide residue of 5 amino acids, Xac represents a peptide residue of 5 amino acids, Xad represents a peptide residue of 3 amino acids, and Xae represents a peptide residue comprising at least 1 amino acid.

[0015] Advantageously, Xab and/or Xad and/or Xae comprise at least one basic amino acid, preferably 1. According to the invention, the term basic amino acids is understood to refer to amino acids chosen from lysine, asparagine and homoasparagine.

[0016] Preferably, Xaa represents the peptide sequence Xaa'-Val, in which Xaa' represents $NH_2$ or a peptide residue comprising at least 1 amino acid, and/or Xab represents the peptide sequence -Arg-Xab'-Ile, in which Xab' represents a peptide residue of 3 amino acids, and/or Xac represents the peptide sequence -Arg-Xac'-Gly-, in which Xac' represents a peptide residue of 3 amino acids, and/or Xad represents the peptide sequence -Tyr-Xad'-Lys, in which Xad' represents a peptide residue of 1 amino acid, and/or Xae represents the peptide sequence -Thr-Xae', in which Xae' represents COOH or a peptide residue comprising at least 1 amino acid.

[0017] Preferably, Xaa' represents the peptide sequence Arg-Ser-, and/or Xab' represents the peptide sequence -Gln-Ile-Lys-, and/or Xac' represents the peptide sequence -Arg-Arg-Gly-, and/or Xad' represents the peptide residue -Tyr-, and/or Xae' represents the peptide sequence -Asn-Arg-Pro-Tyr.

[0018] According to a preferred embodiment of the invention, androctonine is represented by the peptide sequence of 25 amino acids described by SEQ ID NO.:1 and the homologous peptide sequences.

[0019] The term homologous peptide sequence is understood to refer to any equivalent sequence comprising at least 65% homology with the sequence represented by SEQ ID NO.:2, it being understood that the 4 cysteine residues and the number of amino acids separating them remain identical, certain amino acids being replaced with different but equivalent amino acids on sites which do not induce a substantial change in the antifungal or antibacterial activity of the homologous sequence. Preferably, the homologous sequences comprise at least 75% homology, more preferably at least 85% homology and even more preferably 90% homology.

[0020] The $NH_2$-terminal residue of androctonine can exhibit a post-translational modification, for example an acetylation, while the C-terminal residue can exhibit a post-translational modification, for example an amidation.

[0021] The expression peptide sequence essentially comprising the peptide sequence of general formula (I) is understood to refer not only to the sequences defined above, but also to such sequences comprising, at one or other of their ends or at both ends, peptide residues required for their expression and targeting in a host organism, in particular a plant cell or plant.

[0022] This in particular concerns a "peptide-androctonine" or "androctonine-peptide", advantageously "peptide-androctonine", fusion peptide whose cleavage by the enzymatic systems of the plant cells allows the release of the androctonine defined above. The peptide fused to

androctonine can be a signal peptide or a transit peptide which allows the production of androctonine to be controlled and oriented specifically in one part of the host organism, in particular of the plant cell or plant, such as, for example, the cytoplasm or the cell membrane, or in the case of plants, in a specific type of cell or tissue compartment or in the extracelluar matrix.

[0023] According to one embodiment, the transit peptide can be a chloroplast-addressing signal or a mitochondrion-addressing signal, which is then cleaved off in the chloroplasts or the mitochondria.

[0024] According to another embodiment of the invention, the signal peptide can be an N-terminal signal or "prepeptide", optionally in combination with a signal responsible for retaining the protein in the endoplasmic reticulum, or a vacuole-addressing peptide or "propeptide". The endoplasmic reticulum is the site at which maturation operations on the protein produced, such as, for example, cleavage of the signal peptide, are undertaken by the "cell machinery".

[0025] The transit peptides can be single or double, and, in this case, optionally separated by an intermediate sequence, *i.e.* one comprising, in the direction of transcription, a sequence coding for a transit peptide of a plant gene which codes for a plastid localization enzyme, a portion of sequence of the N-terminal mature portion of a plant gene coding for a plastid localization enzyme, and then a sequence coding for a second transit peptide of a plant gene coding for a plastid localization enzyme, as described in patent application EP 0,508,909.

[0026] As transit peptide which is useful according to the invention, mention may be made in particular of the signal peptide of the tobacco PR-1$\alpha$ gene (WO 95/19443), represented with its coding sequence by SEQ ID NO.:2 and fused to androctonine (SEQ ID NO.:3), in particular corresponding to the fusion protein corresponding to bases 12 to 176 of this sequence, in particular when the androctonine is produced by plant cells or plants, or the precursor of Mat $\alpha$1 factor when the androctonine is produced in yeasts.

**[0027]** The present invention thus relates, firstly, to a nucleic acid fragment, in particular a DNA fragment, coding for the androctonine defined above. According to the invention, this can be a fragment isolated from *Androctonus australis*, or alternatively a derived fragment, adapted for the expression of androctonine in the host organism in which the peptide will be expressed. The nucleic acid fragment can be obtained according to the standard methods for isolation and purification, or alternatively by synthesis according to the usual techniques of successive hybridizations of synthetic oligonucleotides. These techniques are described in particular by Ausubel *et al.*

**[0028]** According to the present invention, the expression "nucleic acid fragment" is understood to refer to a nucleotide sequence which can be of DNA or RNA type, preferably of DNA type, in particular cDNA, especially of double-stranded type.

**[0029]** According to one embodiment of the invention, the nucleic acid fragment coding for androctonine is the DNA sequence described by SEQ ID NO.:1, a homologous sequence or a sequence complementary to the sequence, more particularly the coding portion of SEQ ID NO.:1, corresponding to bases 1 to 75.

**[0030]** According to the invention, the term "homologous" is understood to refer to a nucleic acid fragment having one or more sequence modifications when compared with the nucleotide sequence described by SEQ ID NO.:1 coding for androctonine. These modifications can be obtained according to the usual mutation techniques, or alternatively by selecting the synthetic oligonucleotides used in the preparation of the sequence by hybridization. With regard to multiple combinations of nucleic acids which can lead to the expression of the same amino acid, the differences between the reference sequence described by SEQ ID NO.:1 and the homologous sequence can be considerable, and all the more so when it concerns a DNA fragment less than 100 nucleic acids in size, which can be produced by synthesis. Advantageously, the degree of homology will be at least 70% relative to the

reference sequence, preferably at least 80% and more preferably at least 90%. These modifications are generally neutral, *i.e.* they do not affect the primary sequence of the resulting androctonine.

**[0031]** The present invention also relates to a chimeric gene (or expression cassette) comprising a coding sequence and heterologous regulation elements in positions 5' and 3' which can function in a host organism, in particular plant cells or plants, these elements being functionally linked to the coding sequence, the coding sequence comprising at least one DNA fragment coding for androctonine as defined above (including the "peptide-androctonine" or "androctonine-peptide" fusion peptide).

**[0032]** The term host organism is understood to refer to any lower-order or higher-order monocellular or multicellular organism into which the chimeric gene according to the invention can be introduced for the production of androctonine. Such organisms are, in particular, bacteria, for example *E. coli*, yeasts, in particular yeasts of the genera *Saccharomyces* or *Kluyveromyces, Pichia*, fungi, in particular *Aspergillus*, a baculovirus, or, preferably, plant cells and plants.

**[0033]** According to the invention, the term "plant cell" is understood to refer to any plant-derived cell which can constitute undifferentiated tissues such as calli, differentiated tissues such as embryos, plant portions, plants or seeds.

**[0034]** According to the invention, the term "plant" is understood to refer to any differentiated multicellular organism capable of photosynthesis, in particular monocotyledons or dicotyledons, more particularly crop plants which may or may not be intended for human or animal consumption, such as corn, wheat, rapeseed, soybean, rice, sugar cane, beetroot, tobacco, cotton, *etc.*

**[0035]** The regulation elements required for the expression of the DNA fragment coding for androctonine are well known to those skilled in the art as a function of the host organism. They comprise in particular promoter sequences, transcription activators, transit peptides and termination

sequences, including start and stop codons. The means and methods for identifying and selecting the regulation elements are well known to those skilled in the art.

**[0036]** For the transformation of microorganisms such as yeasts or bacteria, the regulation elements are well known to those skilled in the art and comprise, in particular, promoter sequences, transcription activators, transit peptides, termination sequences and start and stop codons.

**[0037]** In order to direct the expression and secretion of the peptide in the yeast culture medium, a DNA fragment coding for heliomycin is incorporated into a shuttle vector which comprises the following elements:

- markers which allow the transformants to be selected,
- a nucleic acid sequence which allows replication (origin of replication) of the plasmid in the yeast,
- a nucleic acid sequence which allows replication (origin of replication) of the plasmid in *E. coli*,
- an expression cassette consisting of
    (1) a promoter regulation sequence,
    (2) a sequence coding for a signal peptide (or prepeptide) combined with an addressing peptide (or propeptide),
    (3) a polyadenylation or terminator regulation sequence.

These elements have been described in several publications, including Reichhart *et al.*, 1992, Invert. Reprod. Dev., 21, pp. 15-24 and Michaut *et al.*, 1996, FEBS Letters, 395, pp. 6-10.

**[0038]** Preferably, yeasts from the species *S. cerevisiae* are transformed with the expression plasmid by the lithium acetate method (Ito *et al.*, 1993, J. Bacteriol, 153, pp. 163-168).

[0039] The invention relates more particularly to the transformation of plants. As promoter regulation sequence in plants, it is possible to use any promoter sequence of a gene which is naturally expressed in plants, in particular a promoter of bacterial, viral or plant origin such as, for example, that of a gene for the small subunit of ribulose biscarboxylase/oxygenase (RuBisCO) or of a plant virus gene such as, for example, that of cauliflower mosaic virus (CAMV 19S or 35S), or a promoter which can be induced by pathogens such as tobacco PR-1a, it being possible to use any suitable known promoter. Preferably, use is made of a promoter regulation sequence which favours the overexpression of the coding sequence in a constitutive manner or induced by the attack of a pathogen, such as, for example, that comprising at least one histone promoter as described in patent application EP 0,507,698.

[0040] According to the invention, it is also possible to use, in combination with the promoter regulation sequence, other regulation sequences which are located between the promoter and the coding sequence, such as transcription activators ("enhancers"), such as, for example, the tobacco mosaic virus (TMV) translation activator described in International Patent Application WO 87/07644, or the tobacco etch virus (TEV) translation activator described by Carrington & Freed (1990).

[0041] As polyadenylation or terminator regulation sequence, it is possible to use any corresponding sequence of bacterial origin, such as, for example, the nos terminator of *Agrobacterium tumefaciens*, or alternatively of plant origin, such as, for example, a histone terminator as described in patent application EP 0,633,317.

[0042] According to the present invention, the chimeric gene can also be combined with a selection marker adapted to the transformed host organism. Such selection markers are well known to those

skilled in the art. Such a marker may be an antibiotic-resistance gene or alternatively a herbicide-tolerance gene for plants.

[0043] The present invention also relates to a cloning or expression vector for the transformation of a host organism containing at least one chimeric gene as defined above. Besides the above chimeric gene, this vector comprises at least one origin of replication and, where appropriate, a suitable selection marker. This vector can consist of a plasmid, a cosmid, a bacteriophage or a virus, which are transformed by introducing the chimeric gene according to the invention. Depending on the host organism to be transformed, such transformation vectors are well known to those skilled in the art and are widely described in the literature.

[0044] For the transformation of plant cells or plants, such a vector is, in particular, a virus which can be used for the transformation of the plants developed and also containing its own replication and expression elements. Preferably, the vector for transforming the plant cells or plants according to the invention is a plasmid.

[0045] The subject of the invention is also a process for transforming host organisms, in particular plant cells, by incorporating at least one nucleic acid fragment or one chimeric gene as defined above, it being possible for this transformation to be obtained by any suitable known means, which is amply described in the specialized literature, and in particular the references cited in the present application, more particularly by means of the vector according to the invention.

[0046] One series of methods consists in bombarding cells, protoplasts or tissues with particles to which the DNA sequences are attached. Another series of methods consists in using, as a means of transfer into the plant, a chimeric gene inserted into a Ti plasmid of *Agrobacterium tumefaciens* or an Ri plasmid of *Agrobacterium rhizogenes*.

[0047] Other methods can be used, such as microinjection or electroporation, or alternatively direct precipitation using PEG.

[0048] A person skilled in the art will select the appropriate method as a function of the nature of the host organism, in particular the plant cell or plant.

[0049] The subject of the present invention is also transformed host organisms, in particular plant cells or plants, containing an effective amount of a chimeric gene comprising a sequence coding for the androctonine defined above.

[0050] The subject of the present invention is also plants containing transformed cells, in particular plants regenerated from the transformed cells. The regeneration is obtained by any suitable process which depends on the nature of the species, as described, for example, in the above references.

[0051] For the processes for transforming plant cells and for regenerating plants, mention will be made in particular of the following patents and patent applications: US 4,459,355, US 4,536,475, US 5,464,763, US 5,177,010, US 5,187,073, EP 267,159, EP 604 662, EP 672 752, US 4,945,050, US 5,036,006, US 5,100,792, US 5,371,014, US 5,478,744, US 5,179,022, US 5,565,346, US 5,484,956, US 5,508,468, US 5,538,877, US 5,554,798, US 5,489,520, US 5,510,318, US 5,204,253, US 5,405,765, EP 442 174, EP 486 233, EP 486 234, EP 539 563, EP 674 725, WO 91/02071 and WO 95/06128.

[0052] The subject of the present invention is also the transformed plants obtained from the cultivating and/or crossing of the above regenerated plants, as well as the seeds of transformed plants.

[0053] The plants thus transformed are resistant to certain diseases, in particular to certain fungal or bacterial diseases. Consequently, the DNA sequence coding for androctonine can be inserted with the main aim of producing plants that are resistant to the diseases, since androctonine is effective

against fungal diseases such as those caused by *Cercospora*, in particular *Cercospora beticola, Cladosporium*, in particular *Cladosporium herbarum, Fusarium*, in particular *Fusarium culmorum* or *Fusarium graminearum*, or by *Phytophthora*, in particular *Phytophthora cinnamomi.*

**[0054]** The chimeric gene may also advantageously be combined with at least one selection marker, such as one or more herbicide-tolerance genes.

**[0055]** The DNA sequence coding for androctonine can also be inserted as a selection marker during the transformation of plants with other sequences coding for other peptides or proteins of interest, such as, for example, herbicide-tolerance genes.

**[0056]** Such herbicide-tolerance genes are well known to those skilled in the art and are described in particular in patent applications EP 115,673, WO 87/04181, EP 337,899, WO 96/38567 or WO 97/04103.

**[0057]** Needless to say, the transformed cells and plants according to the invention can also comprise the sequence coding for androctonine, other heterologous sequences coding for proteins of interest, such as other complementary peptides capable of giving the plant resistance to other diseases of bacterial or fungal origin, and/or other sequences coding for herbicide-tolerance proteins, in particular defined above and/or other sequences coding for insect-resistance proteins, such as the *Bt* proteins in particular.

**[0058]** The other sequences can be inserted using the same vector comprising the chimeric gene according to the invention, which comprises a sequence coding for androctonine, and comprising at least one other gene comprising another sequence coding for another peptide or protein of interest.

**[0059]** They can also be inserted using another vector comprising at least the other sequence, according to the usual techniques defined above.

[0060] The plants according to the invention can also be obtained by crossing parents, one carrying the gene according to the invention coding for androctonine, the other carrying a gene coding for at least one other peptide or protein of interest.

[0061] Among the sequences coding for other antifungal peptides, mention may be made of the one coding for drosomycin, described in patent application Fr 2,725,992 and by Fehlbaum *et al.*, (1994), and in the unpublished patent application FR 97/09115 filed on 24 July 1997.

[0062] Lastly, the present invention relates to a process for cultivating transformed plants according to the invention, the process consisting in planting the seeds of the transformed plants in an area of a cultivation environment, in particular a field, which is suitable for cultivating the plants, in applying an agrochemical composition to the area, without substantially affecting the transformed seeds or plants, and then in harvesting the plants cultivated when they reach the desired maturity, and optionally in separating the seeds from the harvested plants.

[0063] According to the invention, the term agrochemical composition is understood to refer to any agrochemical composition comprising at least one active product having either herbicidal, fungicidal, bactericidal, virucidal or insecticidal activity.

[0064] According to a preferred embodiment of the cultivation process according to the invention, the agrochemical composition comprises at least one active product having at least a fungicidal and/or bactericidal activity, more preferably having an activity complementary to that of the androctonine produced by the transformed plants according to the invention.

[0065] According to the invention, the expression product having activity complementary to that of androctonine is understood to refer to a product having a complementary spectrum of activity, i.e. a product which will be active against attacks by androctonine-insensitive contaminants (fungi, bacteria or viruses), or alternatively a product whose spectrum of activity totally or partially covers

that of androctonine, and whose dose of application will be substantially reduced on account of the presence of the androctonine produced by the transformed plant.

**[0066]** Lastly, cultivation of the transformed host organisms allows the large-scale production of androctonine. The subject of the present invention is thus also a process for preparing androctonine, comprising the steps of cultivating the transformed host organism comprising a gene coding for androctonine as defined above in an appropriate cultivation environment, followed by the extraction and total or partial purification of the androctonine obtained.

**[0067]** The examples below make it possible to illustrate the invention, the preparation of the sequence coding for androctonine, the chimeric gene, the integration vector and the transformed plants. The attached Figures 1 to 5 describe schematic structures of certain plasmids prepared for the construction of the chimeric genes. In these figures, the various restriction sites are marked in *italics*.

## EXAMPLES

### Example 1: Construction of the chimeric genes

**[0068]** All the techniques used below are standard laboratory techniques. The detailed procedures for these techniques are described in particular in Ausubel *et al.*

**pRPA-MD-P: Creation of a plasmid containing the signal peptide for the tobacco PR-1a gene.**

**[0069]** The two complementary synthetic oligonucleotides Oligo 1 and Oligo 2 below are hybridized at 65°C for 5 minutes and then by slowly decreasing the temperature to 30°C over 30 min.

Oligo 1: 5'GCGTCGACGCGATGGGTTTCGTGCTTTTCTCTCAGCTTCCATCTTTCCTTCTTGT GTCTACTCTTCTTCTTTTCC-3' (SEQ ID NO.:7)

Oligo 2: 5'-TCGCCGGCACGGCAAGAGTAAGAGATCACAAGGAAAAGAAGAAGAGTAG ACACAAGAAGGAAAGATGGAAGC-3' (SEQ ID NO.:8)

[0070] After hybridization between Oligo 1 and Oligo 2, the remaining single-stranded DNA serves as a matrix for the Klenow fragment of *E. coli* polymerase 1 (under the standard conditions recommended by the manufacturer (New England Biolabs)) for the creation of the double-stranded oligonucleotide starting from the 3' end of each oligo. The double-stranded oligonucleotide obtained is then digested with the restriction enzymes *SacII* and *NaeI* and cloned in the plasmid pBS II SK(-) (Stratagene) digested with the same restriction enzymes. A clone comprising the region coding for the signal peptide of the tobacco PR-1a gene (SEQ ID NOS.:3 and 4) is thus obtained.

**pRPA-PS-PR1a-andro: Creation of a sequence coding for androctonine fused to the PR-1a signal peptide without an untranscribed 3' region.**

[0071] The two complementary synthetic oligonucleotide sequences Oligo 3 and Oligo 4 are hybridized according to the operating conditions described for pRPA-MD-P.

Oligo 3: 5'-AGGTCCGTGTGCAGGCAGATCAAGATCTGCAGGAGGAGGGGTGG-3' (SEQ ID NO.:9)

Oligo 4: 5'-CCGGATCCGTCGACACGTTCGCCTCGCCGAGCTCAGTATGGCCTGTTAGTGCA CTTGTAGTAGCAACCACCCCTCCTCCTGCAGATCTTGATCTGCC-3' (SEQ ID NO.:10)

[0072] After hybridization between Oligo 3 and Oligo 4, the remaining single-stranded DNA serves as a matrix for the Klenow fragment of *E. coli* polymerase 1 (under the standard conditions recommended by the manufacturer (New England Biolabs)) for the creation of the double-stranded

oligonucleotide starting from the 3' end of each oligo. This double-stranded oligonucleotide containing the portion coding for androctonine (SEQ ID NO. 1) is then cloned directly in the plasmid pRPA-MD-P, which was digested with the restriction enzyme *NaeI*. The correct orientation of the clone obtained is verified by sequencing. A clone comprising the region coding for the PR-1a-androctonine fusion protein, located between the *NcoI* restriction site at the N-terminal end and the *ScaI, SacII* and *BamHI* restriction sites at the C-terminal end (SEQ ID NOS.:5 and 6), is thus obtained.

**pRPA-RD-238: Creation of an expression vector in plants comprising the sequence coding for the PR-1a androctonine fusion protein.**

[0073] The plasmid pRTL-2 GUS, derived from the plasmid pUC-19, was obtained from Dr. Jim Carrington (Texas A&M University, not described). This plasmid, whose schematic structure is represented in Figure 1, contains the duplicated CaMV 35S promoter isolated from cauliflower mosaic virus (CaMV 2x35S promoter; Odell *et al.*, 1985) which directs the expression of an RNA containing a 5' untranslated sequence of tobacco etch virus (TEV 5' UTR; Carrington and Freed, 1990), the *E. coli* β-glucuronidase gene (GUS; Jefferson *et al.*, 1987) followed by the CaMV RNA 35S polyadenylation site (CaMV polyA; Odell *et al.*, 1985).

[0074] The plasmid pRTL-2 GUS is digested with the restriction enzymes *NcoI* and *BamHI* and the main DNA fragment is purified. The plasmid pRPA-PS-PR1a-andro is digested with the restriction enzymes *NcoI* and *BamHI* and the small DNA fragment containing the region coding for the PR-1a-androctonine fusion protein is purified. The two purified DNA fragments are then linked together in an expression cassette in the plants which synthesizes a PR-1a-androctonine fusion protein. The schematic structure of this expression cassette is represented in Figure 2. "PR-1a-androctonine"

represents the region coding for the PR-1a-androctonine fusion protein of pRPA-RD-230. The androctonine is transported to the plant's extracelluar matrix by the action of the PR-1a peptide signal.

**pRPA-RD-195: Creation of a plasmid containing a modified multiple cloning site.**

**[0075]** The plasmid pRPA-RD-195 is a plasmid derived from pUC-19 which contains a modified multiple cloning site. The complementary synthetic oligonucleotides Oligo 5 and Oligo 6 below are hybridized and made double-stranded according to the procedure described for pRPA-MD-P.

Oligo 5: 5'-AGGGCCCCCTAGGGTTTAAACGGCCAGTCAGGCCGAATTCGAGCTCGGTACC CGGGGATCCTCTAGAGTCGACCTGCAGGCATGC-3' (SEQ ID NO.:11)

Oligo 6: 5'CCCTGAACCAGGCTCGAGGGCGCGCCTTAATTAAAAGCTTGCATGCCTGCAGG TCGACTCTAGAGG-3' (SEQ ID NO.:12)

**[0076]** The double-stranded oligonucleotide obtained is then inserted into pUC-19, which was predigested with the restriction enzymes *EcoRI* and *HindIII* and made blunt at the ends using the Klenow fragment of *E. coli* DNA polymerase 1. A vector containing multiple cloning sites to facilitate the introduction of the expression cassettes into an *Agrobacterium tumefaciens* vector plasmid is obtained. The schematic structure of this multiple cloning site is represented in Figure 3.

**pRPA-RD-233: Introduction of the PR-1a-androctonine expression cassette from pRPA-RD-230 into pRPA-RD-195.**

**[0077]** The plasmid pRPA-RD-230 is digested with the restriction enzyme *HindIII.* The DNA fragment containing the PR-1a-androctonine expression cassette is purified. The purified fragment is then inserted into pRPA-RP-195, which was predigested with the restriction enzyme *HindIII* and dephosphorylated with calf intestinal phosphatase.

**pRPA-RD-174: Plasmid derived from pRPA-BL-150A (EP 0,508,909) containing the bromoxynil-tolerance gene from pRPA-BL-237 (EP 0,508,909).**

[0078] The bromoxynil-tolerance gene is isolated from pRPA-BL-237 by means of a PCR gene amplification. The fragment obtained has blunt ends, and is cloned in the pRPA-BL-150A *EcoRI* site, the ends of which were made blunt by the action of Klenow polymerase under standard conditions. An *Agrobacterium tumefaciens* vector which contains the bromoxynil-tolerance gene close to its right-hand end, a kanamycin-tolerance gene close to its left-hand end and a multiple cloning site between these two genes is obtained.

[0079] The schematic structure of pRPA-RD-174 is represented in Figure 4. In this figure, "nos" represents the polyadenylation site of *Agrobacterium tumefaciens* nopaline synthase (Bevan *et al.*, 1983), "NOS pro" represents the *Agrobacterium tumefaciens* nopaline synthase promoter (Bevan *et al.*, 1983), "NPT II" represents the neomycin phosphotransferase gene of the Tn5 transposon of *E. coli* (Rothstein *et al.*, 1981), "35S pro" represents the 35S promoter isolated from cauliflower mosaic virus (Odell *et al.*, 1985), "BRX" represents the nitrilase gene isolated from *K. ozaenae* (Stalker *et al.*, 1988), "RB" and "LB" represent, respectively, the right-hand and left-hand ends of the sequence of an *Agrobacterium tumefaciens* Ti plasmid.

**pRPA-RD-184: Addition of a new, unique restriction site into pRPA-RD-174.**

[0080] The complementary synthetic oligonucleotides Oligo 7 and Oligo 8 below are hybridized and made double-stranded according to the procedure described for pRPA-MD-P.

Oligo 7: 5'CCGGCCAGTCAGGCCACACTTAATTAAGTTTAAACGCGGCCCCGGCGCGC
CTAGGTGTGTGCTCGAGGGCCCAACCTCAGTACCTGGTTCAGG-3' (SEQ ID NO.:13)

Oligo 8: 5'CCGGCCTGAACCAGGTACTGAGGTTGGGCCCTCGAGCACACACCTAGGCGCG
CCGGGGCCGCGTTTAAACTTAATTAAGTGTGGCCTGACTGG-3' (SEQ ID NO.:14)

[0081] The hybridized double-stranded oligonucleotide (96 base pairs) is purified after separation on agarose gel (3% Nusieve, FMC). The plasmid pRPA-RD-174 is digested with the restriction enzyme *XmaI* and the main DNA fragment is purified. The two DNA fragments obtained are then linked together.

[0082] A plasmid derived from pRPA-RD-174 is obtained, comprising other restriction sites between the bromoxynil-tolerance gene and the selection marker kanamycin gene.

[0083] The schematic structure of the plasmid pRPA-RD-184 is represented in Figure 5, in which the terms "nos", "NPT II", "NOS pro", "35S pro", "BRX gene", "RB" and "LB" have the same meanings as in Figure 4.

**pRPA-RD-236: Creation of an *Agrobacterium tumefaciens* vector containing the gene construct coding for androctonine directed towards the extracellular matrix.**

[0084] The plasmid pRPA-RD-233 is digested with the restriction enzymes *PmeI* and *AscI* and the DNA fragment containing the PR-1a-androctonine gene is purified. The plasmid pRPA-RD-184 is digested with the same restriction enzymes. The DNA fragment containing the PR-1a-androctonine expression cassette is then inserted into pRPA-RD-184. An *Agrobacterium tumefaciens* vector containing the sequence coding for the PR-1a-androctonine fusion protein is thus obtained, which leads to the expression of androctonine in the plant's extracellular matrix.

## Example 2: Tolerance to herbicides of transformed tobacco plants.

### 2.1- Transformation

[0085] The vector pRPA-RD-236 is introduced into the *Agrobacterium tumefaciens* strain EHA101 (Hood *et al.*, 1987) carrying the cosmid pTVK291 (Komari *et al.*, 1986). The transformation technique is based on the procedure by Horsh *et al.* (1985).

### 2.2- Regeneration

[0086] Regeneration of the tobacco plant PBD6 (obtained from SEITA France) from foliar explants is carried out on Murashige-Skoog (MS) base medium comprising 30 g/l of sucrose and 200 μg/ml of kanamycin. The foliar explants are taken from plants cultivated in a greenhouse or *in vitro* and regenerated according to the foliar disc technique (Horsh *et al.*, 1985) in three successive steps: the first step comprises induction of the shoots on a medium supplemented with 30 g/l of sucrose containing 0.05 mg/l of naphthylacetic acid (NAA) and 2 mg/l of benzylaminopurine (BAP) for 2 weeks. The shoots formed during this step are then grown for 10 days by cultivating on MS medium supplemented with 30 g/l of sucrose but containing no hormone. Next, the shoots which have grown are taken and cultivated on an MS rooting medium with half the content of salts, vitamins and sugar and containing no hormone. After about 2 weeks, the rooted shoots are placed in a greenhouse.

### 2.3- Tolerance to bromoxynil

[0087] Twenty transformed plants were regenerated and placed in a greenhouse for the construction of pRPA-RD-236. These plants were treated in the greenhouse, at the 5-leaf stage, with aqueous Pardner suspension corresponding to 0.2 kg of bromoxynil active material per hectare.

**[0088]** All the plants showing complete tolerance to bromoxynil are then used in various experiments which show that the expression of androctonine by the transformed plants makes them resistant to fungal attack.

## REFERENCES

Ausubel, F.A. *et al.*, (eds. Greene). Current Protocols in Molecular Biology. Publ. Wiley & Sons.

Bevan, M. *et al.*, (1983). Nuc. Acids Res. **11**:369-385.

Carrington and Freed (1990). J. Virol. **64**:1590-1597.

Ehret-Sabatier *et al.*, (1996). The Journal of Biological Chemistry, **271, 47**, 29537-29544.

Horsch *et al.*, (1985). Science **227**:1229-1231.

Jefferson *et al.*, (1987). EMBO J. **6**:3901-3907.

Komari *et al.*, (1986). J. Bacteriol. **166**:88-94.

Rothstein *et al.*, (1981). Cold Spring Harb. Symp. Quant. Biol. **45**:99-105.

Stalker *et al.*, (1988). J. Biol. Chem. **263**:6310-6314.

Odell, J.T. *et al.*, (1985). Nature **313**:810-812.